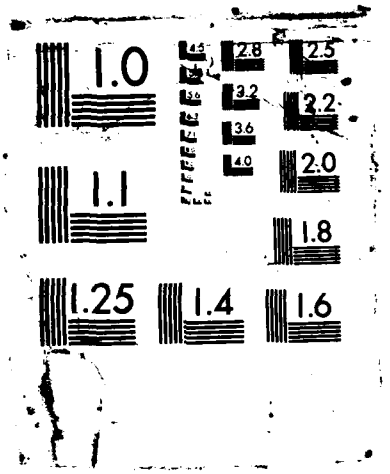
AD-A192 439 THE ROLE OF CHEMICAL INHIBITION OF GAP JUNCTIONAL INTERCELLULAR COMMUNICA..(U) MICHIGAN STATE UNIV EAST LANSING DEPT OF PEDIATRICS/HUMAN DEV.. J E TROSKO 1/1

UNCLASSIFIED 14 FEB 88 AFOSR-TR-88-0248 AFOSR-86-0084 F/G 6/1 NL

END
DATE FILMED
8-


1.0
2.8
2.5
3.2
2.2
3.6
4.0
2.0
1.1
1.8
1.25
1.4
1.6

SECURITY CLASSIFICATION OF THIS PAGE

(2)

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS: None
	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited.
AD-A192 439	5. MONITORING ORGANIZATION REPORT NUMBER(S): AFOSR-TR- 88-0248
6a. NAME OF PERFORMING ORGANIZATION: Michigan State University College of Human Medicine	6b. OFFICE SYMBOL (If applicable):
6c. ADDRESS (City, State, and ZIP Code): Department of Pediatrics/Human Development East Lansing, Michigan 48824	7a. NAME OF MONITORING ORGANIZATION: Air Force Office of Scientific Research/NL
	7b. ADDRESS (City, State, and ZIP Code): Building 410 Bolling AFB, DC 20332-6448
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: AFOSR	8b. OFFICE SYMBOL (If applicable): NL
	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: AFOSR-86-0084
8c. ADDRESS (City, State, and ZIP Code): Building 410 Bolling AFB, DC 20322-6448	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102F	2312	AS	

11. TITLE (Include Security Classification)

The role of chemical inhibition of gap-junctional intercellular communication in toxicology

12. PERSONAL AUTHOR(S)
TROSKO, JAMES E.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Technical Report	FROM 2/15/87 - 2/14/88	02/03/88	11

16. SUPPLEMENTARY NOTATION
Two reprints.

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Gap junctions, cell communication, tumor promoters, teratogens, neurotoxins, protein kinase C, chemical toxicity.
0620			

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Our goal has been to study the mechanism by which non-genotoxic chemicals act. To this end, we are testing the hypothesis that chemical modulation of gap junctional intercellular communication can lead to many toxic endpoints, such as teratogenesis, tumor promotion, immune-, reproductive and neurotoxicities. Our aims have been (a) to study the biochemical mechanisms by which inhibitors of gap junctions work; (b) to develop and apply new in vitro techniques to measure gap junction function; and (c) to test if known non-genotoxic chemicals inhibit gap junctions in various cell types. Results to date have validated the "fluorescence recovery after photobleaching" and scrape-loading/dye transfer techniques for measuring gap junction function. In addition, we have shown that protein kinase C, the ras oncogene and the neurotoxicant, heptachlor, all seem to work via different mechanisms to block intercellular communication. Results described in this report have been communicated at several meetings, while abstracts, preprints and reprints of these reports are attached to the progress report.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
James E. Trosko, Ph.D.	517/353-6346	

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

08 FEB 1988

Annual Technical Report

AFOSR-86-0084
2/15/87 - 2/~~15~~14/88

"The Role of Chemical Inhibition of Gap Junctional Intercellular Communication in Toxicology"

James E. Trosko, Ph.D.
Department of Pediatrics/Human Development
Michigan State University
East Lansing, Michigan 48824

1. SUMMARY

Toxic chemicals exert their actions via several distinct cellular endpoints, mutagenesis, cytotoxicity or alterations of gene expression by modulating gap junctional intercellular communication. Intercellular communication, mediated by the membrane structure, the gap junction, maintains homeostatic control of cell functions (i.e., cell growth, differentiation, or control of differentiated functions), within tissues via the transfer of ions and small molecules. Disruption of this fundamental biochemical process would be expected to lead to a variety of toxicological endpoints. Our research goal has been to test this hypothesis, namely, that chemical modulation of gap junctional intercellular communication can lead to teratogenesis, tumor promotion, reproductive-, immune- and neurotoxicities.

To date, after two years into the project, we have initiated work on all of the specific aims of the original proposal. Using three of our newly developed techniques to measure gap junctional communication (FRAP analysis; scrape-loading/dye transfer and flow cytometry-scrape loading/dye transfer), we have provided new information on the mechanisms by which various chemical inhibitors of gap junctions work (i.e., some by activating protein kinase C; some by altering intracellular calcium levels; others by causing free radical damage of membrane components); on how certain neurotoxins and reproductive toxins work (i.e., heptachlor, heptachlor epoxide; JP-4 jet fuel); and how certain oncogenes (i.e., ras) work to be tumorigenic. We have also attained preliminary results in isolating several "intercellular communication-deficient" mutant cell lines, which have the potential of allowing us to use somatic cell genetics to study the biochemical mechanisms regulating intercellular communication.

2. RESEARCH OBJECTIVES

The original objective of the proposal, namely to study the basic mechanisms controlling gap junctional intercellular communication in mammalian cells, has not changed. To meet this objective, several aims were proposed and work on them has been initiated.

1. To test the hypothesis that inhibition of intercellular communication plays a role in the toxicities of many chemicals.

2. To develop and apply new techniques to measure gap junctional intercellular communication.

3. To study the role of protein kinase C in the regulation of gap junction functions.

4. To isolate gap junction proteins for the production of antibodies to be used to study gap junction function.

5. To study non-protein kinase C mechanisms of chemical inhibition of gap junction function.

3. STATUS OF RESEARCH

After two years, with the powerful means to measure gap junction function, we have finally succeeded in building a "critical mass" of expertize and techniques to study toxic chemicals which act by inhibiting intercellular communication.

Aim 1. To test the hypothesis that inhibition of gap junctional communication plays a role in the consequences of toxic chemicals.

a. A major theoretical assessment has been made illustrating the need to incorporate the mechanism of chemical inhibition of gap junction function in the risk assessment models for exposure to toxic chemicals. This paper will appear in the Banbury Conference Report, Scientific Basis for Qualitative and Quantitative Risk Assessment of Chemical Exposure, R.W. Hart and R.B. Setlow, eds., Cold Spring Harbor, in press.

b. We have completed an in vitro/in vivo comparative study showing that known promoters of rat liver tumors, phenobarbital and polybrominated biphenyls, also inhibited intercellular communication in vitro, using rat liver epithelial cells. In addition to showing a no-effect phenomenon for both of these chemicals; indicating a "threshold" phenomenon for inhibitors of intercellular communication, we studied the comparative utility of the new "FRAP" (Fluorescence Recovery After Photobleaching) technique to that of the older technique of metabolic cooperation. Results demonstrated the advantages and differences between these two techniques. A manuscript is being prepared for submission for publication.

c. A major observation was made showing that two known reproductive toxicants, gossypol (an extract from cotton seed oil and a male anti-fertility dury) and heptachlor, inhibited gap junctional intercellular communication in rat leydig cells. Manuscripts are now being prepared for submission for publication.

d. We have obtained some preliminary data on the jet fuel, JP-4, as a potential inhibitor of intercellular communication. An aqueous extraction of JP-4 jet fuel was prepared by combining the water fractions after 4 extractions of JP-4 with equal parts of water. Various amounts of the JP-4 aqueous extractions were added to subconfluent tissue culture dishes containing rat liver WB cells. After one hour, gap junctional communication was quantitated using the Meridian

ACAS 470. Only at the higher volumes of JP-4 extract (50 and 100 ul) were there any changes in intercellular communication in the liver cells.

Aim 2. To develop and apply new techniques to measure gap junctional intercellular communication.

a. Now that we have developed three new techniques to measure gap junction function, it was necessary to validate, by comparative analysis, each technique to accepted techniques and to each other. This effort has led to two projects which now have been accepted for publication. One has shown, using purified polybrominated biphenyls, that the FRAP technique could measure a dose response effect (including a "threshold" phenomenon) in rat liver cells similar to the metabolic cooperation assay. This work will appear in Cell Biology and Toxicology, 1988.

The other study combined the "FRAP" technique with the scrape-loading/dye transfer assay to be able to quantitate, on a single cell basis, the amount of chemical inhibition of gap junctional intercellular communication. This work will appear in the J. Toxicol. Environ. Health, 1988.

b. The human keratinocyte assay we were working on has now been completed, with a manuscript having been submitted for publication. The assay gives us a normal human in vitro skin system to screen and study potential skin irritants which act by inhibiting intercellular communication.

Aim 3. To study the role of protein kinase C in the inhibition of intercellular communication. Using several inhibitors to protein kinase C [palmitoyl carnitine or 8-N,N-(diethylamino)octyl-3,4,5-trimethyloxy benzoate], we were able to link the activation of protein kinase C with the inhibition of intercellular communication for phorbol ester-type of tumor promoters. This is of significance because if a given toxicant works through the blockage of intercellular communication by activating protein kinase C, one should be able to ameliorate the toxic effect by pre or simultaneous treatment with inhibitors to protein kinase C. Manuscript will appear in Carcinogenesis, in press, 1988.

Aim 4 Isolation of gap junction proteins for the production of antibodies.

We have resummed this major project. During the last six months we have grown large amounts of rat liver

cells in vitro to provide the gap junction protein starting material. Shortly, we will purify the gap junction protein(s) and start making polyclonal antibodies to the gap junction.

Aim 5. To study ron-protein kinase C mechanisms of chemical inhibition of gap junction function.

a. Using heptachlor and heptachlor epoxide as model compounds which are known tumor promoters, neurotoxicants, and reproductive toxicants, we have shown that they inhibit gap junction function without activating protein kinase C. During this study we have developed new techniques utilizing the Meridian ACAS 470 to detect, quantitatively on a single cell basis, intracellular free calcium and free radical production. Using these new techniques, we showed that induced intracellular free calcium was probably responsible for the heptachlor's ability to inhibit gap junction function.

b. One of the most important tool to understand biochemical mechanisms by which gap junctions are regulated is to isolate mutants which are unable to have functional gap junctions. We have succeeded in starting this approach by developing a new strategy for isolating such mutants in rat liver epithelial cells. We are now in the process of genetically characterizing these mutants. Once a good collection of mutants are available, we will then use them to determine how many genes and gene products control gap junction function.

c. One of the approaches to study gap junction function and its role in a toxic endpoint such as cancer is to study various oncogenes on gap junction function. We have now clearly shown that the "ras" oncogene does inhibit gap junction function, by, as yet, a unknown biochemical mechanism. A manuscript has been submitted for publication.

4. List of Published Manuscripts and Preprints

S. Suter, J.E. Trosko, and A. Koestner, "Fluorescence photobleaching assay of dieldrin inhibition of gap junctional intercellular communication in rat glial cells." Fund. Appl. Toxicol., 9:785-794, 1987.

J.E. Trosko, C. Jone, and C.C. Chang, "Inhibition of gap-junctional-mediated intercellular communication, in vitro, by aldrin, dieldrin and toxaphene: A possible cellular mechanism for their tumor-promoting and neurotoxic effects." Molecular

Toxicol., 1:83-93, 1987.

C. Jone, L. Erickson, J.E. Trosko, and C.C. Chang, "Effect of biological toxins on gap-junctional intercellular communication in Chinese hamster V79 cells." Cell Biol. & Toxicol., 3:1-15, 1987.

T.J. Kavanagh, C.C. Chang, and J.E. Trosko, "Effect of the polybrominated biphenyls, Firemaster BP-6,2,2',4,4',5,5'-hexabromobiphenyl, 3,3',4,4',5,5'-hexabromobiphenyl, and 3,3',4,4'-tetrabromobiphenyl on cell-cell communication in cultured human teratocarcinoma cells." Fund. Appl. Toxicol., 8:127-131, 1987.

M.H. El-Fouly, J.E. Trosko, and C.C. Chang, "Scrape-loading and dye transfer: A rapid and simple technique to study gap junctional intercellular communication." Exp. Cell Res., 168:422-430, 1987.

T.J. Kavanagh, P.S. Rabinovitch, M. El-Fouly, J.E. Trosko, C.C. Chang, and G.M. Martin, "Use of flow cytometry and scrape-loading/dye transfer as a rapid quantitative measure of intercellular communication in vitro. Cancer Res., 47:6046-6051, 1987.

R. Loch-Caruso, I.A. Corcos, and J.E. Trosko, "Inhibition of metabolic cooperation by soluble metal compounds." Submitted for publication.

J.E. Trosko and C.C. Chang, "Chemical and oncogene modulation of gap junctional intercellular communication." In: Tumor Promoters: Biological Approaches for Mechanistic Studies and Assay Systems: R. Lagenbach and E. Elmore, eds., Raven Press, N.Y., in press.

J.E. Trosko, "Mechanisms of tumor promotion: Possible role of inhibited intercellular communication." Eur. J. Cancer Clinical Oncol., 23:599-601, 1987.

C. Jone, J.E. Trosko, and C.C. Chang, "Characterization of a rat liver epithelial cell line to detect inhibitors of metabolic cooperation. In Vitro, Cellular and Devel. Biol., 23:214-220, 1987.

J.E. Trosko, C.C. Chang, and B.V. Madhukar, "Chemical and oncogene modulation of intercellular communication in tumor promotion." In: Advances in Modern Environmental Toxicology, H.A. Milman and E. Elmore, eds., Princeton Scientific Pub., Princeton, N.Y., in press.

B.V. Madhukar, J.E. Trosko, and C.C. Chang, "Chemical, oncogene and growth factor modulation of gap junction communication in carcinogenesis." In: Cell Interactions and Gap Junctions, N. Sperelakis and W.C. Cole, eds., CRC Press, Boca Raton, FL, in press.

M.G. Evans, M.H. El-Fouly, J.E. Trosko, and S.D. Sleight, "Anchored cell analysis/sorting coupled with the scrape-loading/dye transfer technique to quantity inhibition of gap junctional intercellular communication in WB-F344 cells by 2,2',4,4',5,5'-hexabromobiphenyl."

M.G. Evans and J.E. Trosko, "Concentration/response effect of 2,2',4,4',5,5'-hexabromobiphenyl on cell-cell communication in vitro: Assessment by fluorescence redistribution after photobleaching ("FRAP"). Cell Biology Toxicol., in press.

S.Y. Oh, B.V. Madhukar, and J.E. Trosko, "Inhibition of gap junctional blockage by palmitoyl carnitine and TMB-8 in a rat liver epithelial cell line. Carcinogenesis, in press.

M.S. Rezabek, J.E. Trosko, C. Jone, and S.D. Sleight, "Effects of hepatic tumor promoters, phenobarbital and polybrominated biphenyls, on intercellular communication between rat liver epithelial cells, in preparation.

M.H. El-Fouly, J.E. Trosko, C.C. Chang, and S.T. Warren, "The human H-ras oncogene inhibits gap junctional intercellular communication." Submitted for publication.

J.E. Trosko and C.C. Chang, "Non-genotoxic mechanisms in carcinogenesis: Role of inhibited intercellular communication." In: New Directions in the Qualitative and Quantitative Aspects of Carcinogen Risk Assessment, R. Battey, ed., Cold Spring Harbor Laboratory Press, NY, in press.

J.E. Trosko, C.C. Chang, B.V. Madhukar, S.Y. Oh, D. Bombick, and M. El-Fouly, "Modulation of gap junction intercellular communication by tumor promoting chemicals, oncogenes and growth factors during carcinogenesis." In: Gap Junctions, E. Hertzberg and R. Johnson, eds., Alan L. Liss, Inc., in press.

B.V. Madhukar, S.Y. Oh, C.C. Chang, M. Wade, M.H. El-Fouly, and J.E. Trosko, "Altered regulation of intercellular communication by epidermal growth factor, transforming growth factor- and peptide hormones in normal human keratinocytes." Submitted.

5. Professional Personnel

J.E. Trosko, Ph.D., Professor of Pediatrics/Human Development, College of Human Medicine, Center for Environmental Toxicology, Michigan State University, Principal Investigator.

B.V. Madhukar, Ph.D., Assistant Research Professor, Department of Pediatrics/Human Development.

M.H. El-Fouly, Graduate Student, M.D. from University of Alexandria, Egypt and M.S. from University of Michigan, 1984.

Saw Yin Oh, Ph.D., Research Associate, Department of Pediatrics/Human Development, (Replaces Laurie Parker).

6. Interactions

A. Spoken papers.

1. M.H. El-Fouly and J.E. Trosko, "Scrape-loading and dye transfer: A rapid and simple technique for the detection of intercellular communication." Tissue Culture Assoc. Mtg., Chicago, June 4-8, 1986.

2. J.E. Trosko, C.C. Chang, M.H. El-Fouly, R. Kulkarni, and R. Gera, "Role of gap junctional communication in normal and malignant human epithelial cells." Intern. Cell and Tissue Culture Confer., Hershey, PA, Spet. 25, 1986.

3. M.H. El-Fouly, S.T. Warren, J.E. Trosko, and C.C. Chang, "Inhibition of gap junction-mediated intercellular communication in cells transfected with the human H-ras oncogene." Amer. Soc. Human Genetics Mtg., Philadelphia, PA, November 2-5, 1986.

4. J.E. Trosko (seminar), "Adaptive and nonadaptive consequences of chemical inhibition of intercellular communication." Columbia University College of Physicians and Surgeons, April 7, 1986. [Host, Dr. Carmia Borek].

5. J.E. Trosko (seminar), "Oncogenes, intercellular communication, and carcinogenesis." Dept. Pathology, New York University Medical Center, April 8, 1986. [Host, Dr. Angel Pellicer].

6. J.E. Trosko (seminar/consultant), "New methods to detect chemical inhibitors of intercellular communication." R.J. Reynolds/Nabisco Laboratory, Winston-Salem, NC, May, 1986. [Host, Dr. Dave Doolittle].

7. M.H. El-Fouly and J.E. Trosko (poster), "Role of chemical inhibition of cell-cell communication in toxicology." July 31, 1986. [Organizer, Dr. Steve Aust].

8. J.E. Trosko, "Chemical and oncogene modulation of gap junctional intercellular communication." NIEHS Conference, "Tumor Promoters: Biological approaches for mechanistic studies and assay systems." Research Triangle Park, NC, Sept. 8-10, 1986. [Organizer, Dr. R. Lagenbach].

9. J.E. Trosko (symposium speaker), "The role of inhibition of DNA polymerase in DNA amplification in Chinese hamster cells." Deutscher Krebsforschungs-zentrum,

Heidelberg, Germany, Oct. 24-26, 1986.

10. J.E. Trosko (symposium talk), "Role of intercellular communication on aging." University-Based Research on Aging, Michigan State University, Nov. 11, 1986.

11. J.E. Trosko (seminar speaker), "Pharmacological and toxicological effects of chemical modulation of gap junction function." Dept. Pharmacology and Toxicology, M.S.U., Nov. 18, 1986.

12. J.E. Trosko (seminar speaker), "Inhibition of gap junctional communication by chemicals and oncogenes during carcinogenesis. Boston University School of Medicine, Dec. 4, 1986.

13. J.E. Trosko (seminar speaker), "Oncogenes, inhibition of intercellular communication and tumor promotion." Emory University School of Medicine, Atlanta, Dec. 12, 1986.

14. J.E. Trosko (seminar speaker), "Oncogenes, inhibition of intercellular communication and tumor promotion: An integrated hypothesis involving inhibition of gap junctional communication." University of Texas Cancer Center, Science Park, Texas, April 14, 1987.

15. J.E. Trosko (symposium speaker), "Oncogenes, growth factor and tumor promoter modulation of gap junctional communication." Internatonal Conference on Gap Junctions, Asilomar Conference Center, Pacific Grove, California, July 6-10, 1987.

16. J.E. Trosko (symposium speaker), "Non-genotoxic mechanisms in carcinogenesis: Role of inhibited intercellular communication." Banbury Conference, Cold Spring Harbor, NY, Oct. 11, 1987.

17. J.E. Trosko (symposium speaker), "Chemical modulation of gap junctional communication: Implications for varied toxicological responses." Northeast Regional Society of Toxicology Mtg., Boston, Oct. 23, 1987.

18. J.E. Trosko (lecturer), "Oncogenes, chemical tumor promoters and growth factors: An integrated hypothesis for carcinogenesis." Univ. of Maryland School of Medicine, Dept. of Pathology, Nov. 12, 1987.

19. J.E. Trosko (seminar speaker), "Oncogenes, chemical tumor promoters and gap junctions: An integrated theory of carcinogenesis. Frederick Cancer Res. Facility, Dec. 9, 1987.

20. J.E. Trosko (invited speaker), "Towards understanding carcinogenic hazards: A crisis in paradigms."

Brookings Institute, Wash., D.C., Jan 12, 1988.

21. J.E. Trosko (course lecturer), "Role of chemical inhibition of intercellular communication in toxicology." Corriell Instute, Camden, NJ, Jan. 14, 1988.

22. J.E. Trosko (seminar speaker), "Epigenetic toxicology: Role of chemical modulation of intercellular communication in toxicology." Univ. of Connecticut, Storrs, Jan. 15, 1988.

23. J.E. Trosko (distinguished faculty lecturer), "An integrative theory of carcinogenesis: Chemical, oncogene and growth factor inhibition of intercellular communication." Medical College of Ohio, Toledo, OH, Jan. 20, 1988.

24. J.E. Trosko (symposium speaker), "Dysfunctional intercellular communication: Implications to the cause and cure of cancer." Tampa, FL, Jan 25, 1988.

25. J.E. Trosko (seminar speaker), "Role of epigenetic mechanisms in carcinogenesis: Implications for risk assessment." Oak Ridge National Laboratory, Jan. 27, 1988.

26. J.E. Trokso (symposium speaker), "Role of inhibited intercellular communication in tumor promotion." Hawaii, Feb. 9-14, 1988.

7. New Discoveries, Inventions, Patent Disclosures and Specific Applications.

The work contained in the last 6 months of this second year of the grant has re-inforced the original hypothesis that chemical inhibition of gap junctional communication plays a major role in non-genotoxic toxicology ("Epigenetic toxicology"). In fact, I have recently coined this term, "Epigenetic toxicology" to be distinguished from "genetic toxicology". In addition, the recent work continues to validate the three new in vitro assays to measure gap junction function. This should now allow us to understand the mechanisms by which non-genotoxic chemicals work and to allow us better means to predict for risk assessment purposes the potent toxic effects of chemicals using in vitro, rather than animal models.

8. Additional Statements Regarding State of Project.

The significance of this AFOSR supported research can be viewed in light of the fact that an international conference on "The role of chemical inhibition of gap junction intercellular communication in toxicology" will be held in September, 1988. In addition, as evidenced by both the increased frequency of invited talks I have been asked to

give on this research and on our accepted research publications, it is now apparent that our ideas on the importance of this area of toxicology is being widely accepted.

On the level of the laboratory, I will be loosing Mohamed El-Fouly, my graduate student, who will complete his Ph.D. I therefore will be needing to replace him on the project. Also, the need for an upgrade of the Meridian ACAS 470 instrument is critical, since we have developed new applications for this instrument (using the Meridian Company's instrument) for the study of mechanisms (e.g., Ca^{+}+, pH and free radical quantitative determinations).

FUNDAMENTAL AND APPLIED TOXICOLOGY 9, 785–794 (1987)

Dieldrin Inhibition of Gap Junctional Intercellular Communication in Rat Glial Cells as Measured by the Fluorescence Photobleaching and Scrape Loading/Dye Transfer Assays[1]

S. SUTER,* J. E. TROSKO,*[,2] M. H. EL-FOULY,* L. R. LOCKWOOD,* AND A. KOESTNER†

*Center for Environmental Toxicology and Department of Pediatrics and Human Development and †Department of Pathology, Michigan State University, East Lansing, Michigan 48824

Dieldrin Inhibition of Gap Junctional Intercellular Communication in Rat Glial Cells as Measured by the Fluorescence Photobleaching and Scrape Loading/Dye Transfer Assays. SUTER, S., TROSKO, J. E., EL-FOULY, M. H., LOCKWOOD, L. R., AND KOESTNER, A. (1987). *Fundam. Appl. Toxicol.* **9**, 785–794. Application of the fluorescence-recovery after photobleaching (FRAP analysis) technique and scrape loading/dye transfer assay was made to measure the presence of gap junctional communication in primary rat glial cells *in vitro* in the presence and absence of the neurotoxicant and tumor promoter dieldrin, a chlorinated insecticide. Results demonstrate that primary rat glial cells are able to exhibit gap junctional intercellular communication and that dieldrin at noncytotoxic concentrations can modulate gap junctional communication as early as 10 min after exposure to the chemical and that the effect is reversible after 4 hr recovery from the dieldrin exposure. Both the FRAP analysis and the scrape loading/dye transfer assay have validated the observation that dieldrin inhibits gap junctional communication in other cell types using different techniques to measure gap junction function. These results were interpreted as an indication that inhibition of gap junctional communication might contribute to the cellular mechanism of dieldrin's neurotoxicity.

Gap junctional-mediated intercellular communication has been regarded as an important determinant for homeostasis in organisms composed of functionally specialized cells for normal cell growth and differentiation, reproductive, neuroendocrine, and cardiac function, and a whole host of other normal physiological states (Bennett and Goodenough, 1978; Loewenstein, 1979; Hertzberg *et al.*, 1981; Pitts, 1980; Bennett *et al.*, 1981; Schultz, 1985; Larsen, 1983). Low-molecular-weight substances (≦1500 MW) can be transported from cell to cell via gap junctions on contiguous cells (Loewenstein, 1979). Disruption of gap junctional intercellular communication has been postulated to play a role in carcinogenesis (Loewenstein and Kanno, 1966), specifically during the tumor-promotion phases (Yotti *et al.*, 1979; Murray and Fitzgerald, 1979; Trosko *et al.*, 1983). In addition, many tumor-promoting chemicals (Jone *et al.*, 1985) and a few oncogenes (Chang *et al.*, 1985; Azarnia and Loewenstein, 1984; Atkinson and Sheridan, 1984; Atkinson *et al.*, 1986; Azarnia and Loewenstein, 1987) have been associated with inhibited intercellular communication.

Gap junctional intercellular communication has been measured by a variety of techniques, including electrocoupling (Furshpan

[1] Research was sponsored, in part, by a grant from the College of Human Medicine and by the Air Force Office of Scientific Research, Air Force Systems Command, USAF, under Grant No. AFOSR-86-0084, to J. E. Trosko and by a National Institute of Health Grant (CA32594) to A. Koestner.

[2] To whom all correspondence should be addressed at Department of Pediatrics and Human Development, Michigan State University, B240 Life Science Building, East Lansing, MI 48824.

0272-0590/87 $3.00

and Potter, 1959); intercellular transfer of injected fluorescent dyes (Loewenstein, 1966); use of genetically deficient cells to measure "metabolic cooperation" (Hooper, 1982; Davidson *et al.*, 1985; Gupta *et al.*, 1985); and autoradiographic detection of the transfer of low-molecular-weight radioactive labeled compounds (Subak-Sharpe *et al.*, 1969). The ultrastructural analysis of gap junctions is performed by freeze–fracture analysis of cell membranes (Finbow and Yancey, 1981; Larsen, 1983; Larsen and Risinger, 1985). Recently, two new techniques, one using fluorescence-recovery after photobleaching (FRAP analysis), and the other, the scrape loading/dye transfer assay, have been applied to measure gap junctional intercellular communication (Wade *et al.*, 1986; El-Fouly *et al.*, 1987).

Dieldrin, belonging to the cyclodiene class of chlorinated insecticides, is a well-documented toxic chemical. It has been found to be carcinogenic in laboratory rodents, specifically it seems to act as a tumor promoter (Ito *et al.*, 1980; Tennekes *et al.*, 1982). Similar to 12-tetradecanyolphorbol-13-acetate (TPA), dieldrin has been shown to be nonmutagenic in most genotoxic assays (McCann *et al.*, 1975; Ashwood-Smith, 1981; Purchase *et al.*, 1978; Probst *et al.*, 1981; Tong *et al.*, 1981; ICPEMC, 1984). On the other hand, dieldrin has been shown to inhibit metabolic cooperation (a form of gap junctional communication) in Chinese hamster V79 cells (Trosko *et al.*, 1987) and human teratocarcinoma cells (Lin *et al.*, 1986). In addition, dieldrin is known to be a neurotoxin (Joy, 1982). Since gap junctions are known to exist in neuroectodermal cells, this study was designed to determine if some of the neurotoxic effects of dieldrin might be related to its ability to inhibit gap junctional intercellular communication.

MATERIALS AND METHODS

Cells. Normal rat glial cells were subcultured from primarily cultured rat glial cells isolated from cerebral tissue of rat fetuses at the 20th gestation day (Ko *et al.*, 1980). Cells within 10 passages were grown in modified Eagle's medium (MEM; GIBCO formulas 78-5470; Earle's balanced salt solution with 50% increase of vitamins and essential amino acids except glutamine), supplemented with nonessential amino acid (100% increase), 1 mM sodium pyruvate, and 10% fetal calf serum. Under the incubation condition with 5% CO_2 in humidified air at 37°C, cells growing in monolayer, contact-inhibited upon confluency, were subcultured every 5 to 7 days.

Chemicals. 5 (and 6)-Carboxyfluorescein diacetate and rhodamine lissamine dextran (Lot 5B) were obtained from Molecular Probes (Eugene, OR). Lucifer yellow CH was from Sigma Chemical Co. (St. Louis, MO). Dieldrin [Shell Chemical Co. (purity 99+%)] was a gift from Dr. B. V. Madhukar of the Pesticide Research Center at Michigan State University.

Methods. Experiments were performed with rat glial cells plated in the modified MEM. Dieldrin, dissolved in ethyl alcohol (ETOH), was added to cells for various lengths of time to give a final concentration of 7 μg/ml of medium (0.1% final concentration of ETOH). An identical volume of ethyl alcohol, the solvent carrier, was added to the control cells. Neither the solvent carrier nor the carrier plus dieldrin was cytotoxic to the cells at this concentration nor did the concentration of ETOH interfere with intercellular communication.

To measure gap junctional communication using the FRAP analysis technique, following 24 hr of growth, the cells were washed with PBS containing calcium (0.9 mM) and magnesium (0.5 mM; PBS/Ca/Mg) and stained with 6-carboxyfluorescein diacetate. The dye and labeling conditions do not affect cell viability, and restaining can be performed on the same cells for several days. All measurements are performed at room temperature in PBS/Ca^{2+}/Mg^{2+} within a 1-hr period. A tissue culture plate of labeled cells is placed on a high-speed computer-controled two-dimensional stage of the ACAS 470 workstation (Wade *et al.*, 1986). The Meridian ACAS 470 (Anchored Cell Analysis and Sorting, Meridian Instruments, Okemos, MI) was the standard instrument which was equipped with a 2-W argon ion laser tuned to the 488-nm line, dichroic filter at 510 nm and barrier filter at 520 nm, inverted phase-contrast microscope and 16-bit microcomputer for data acquisition and processing, and micro-stepping stage. The stage moves the cells in a defined manner above the objective (40×) of an inverted epifluorescence microscope. The microscope objective serves to focus the argon laser beam (excitation wave length of 488 nm) to a 1-μm spot size that excites fluorescence in individual cells at 1.5-μm steps in a two-dimensional raster pattern. The single-point emission from each excited step is recorded as an intensity by a photomultiplier tube. The digital signals representative of fluorescence intensity are stored in the computer with the source x–y location. The emitted intensities are color

coded and presented on a computer video screen as a pseudo-color image of the fluorescence distribution in the analyzed cell.

In order to measure gap junctional communication by another independent method, the scrape loading/dye transfer assay was utilized. Rat glial cells were subcultured using trypsin (0.01%) without EDTA and plated to attain a confluent monolayer ($1.5–2.0 \times 10^6$ cells) in 35-mm plastic dishes. The cells were incubated in the modified Eagle's medium with 5% FCS at 37°C in humidified air with 5% CO_2 for 12–18 hr. Six plates were prepared for each experimental point including untreated controls and controls with solvent (0.1% absolute ethanol final concentration) only. For temporal studies, the cells were treated with a single dose of dieldrin for various exposure times (6, 10, 15, 20, 30, 50, and 60 min and 24 hr). This predetermined noncytotoxic dose of 7 μg/ml has been previously shown to induce a complete blockage of gap junctional intercellular communication and dye transfer in rat glial cells (El-Fouly *et al.*, 1987). In addition, to test for the reversibility of the dieldrin effect on gap junction conductance following a short-term exposure, the cells were treated with dieldrin (7 μg/ml) for 1 hr then washed with PBS and reincubated after the addition of fresh media for 24 hr.

For dose–response experiments, dieldrin was added to each plate at various noncytotoxic concentrations (1, 2, 3, 5, 6, 7, and 10 μg/ml) for a fixed 2-hr exposure time prior to scrape loading.

In preparation for scrape loading/dye transfer, the cells were washed with PBS (kept at room temperature), then exposed to a dye mixture containing 0.05% of each of Lucifer yellow (MW 457.2) and rhodamine lissamine dextran (MW 10,000) dissolved in PBS. The dye molecules were loaded intracellularly by scraping or cutting the cells using a wooden probe or a sharp knife. The dye solution was left on the cells for 90 sec, then discarded, and the plates were carefully rinsed in PBS to minimize the background fluorescence. The cells were next examined for dye transfer under an inverted Nikon epifluorescence phase microscope with uv light generated from an Osram HBO 200-W bulb. The degree of communication was assessed by measuring the extent of Lucifer yellow transfer into contiguous cells. Quantitation was estimated by counting the number of secondary recipient cells in a fixed surface area selected at random. Ten different fields were examined per plate, six plates per treatment, and an average count is reported as a relative percentage compared to control plates which were considered to have 100% communication.

RESULTS

The effect of dieldrin on the colony-forming ability is shown in Fig. 1. Results show

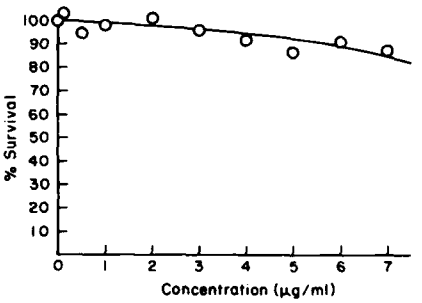


FIG. 1. Effect of dieldrin on the colony-forming ability of primary rat glial cells. The plating efficiency was 87%. Five plates per dose level were counted.

that after a 3-day exposure, even at the highest concentration (7 μg/ml), very little effect was noted in terms of inhibition of the plating efficiency and formation of colonies. It must be noted that the cytotoxicity assay is performed at very low cell densities (200 cells/60-mm plate), and long exposures to the chemical (3 days), whereas the effect of dieldrin on gap junctional communication is done on high densities of cells for short periods of time. Therefore, these cytotoxicity data would be considered overestimates of the effective cytotoxic levels. In other words, dieldrin at 7 μg/ml (or up to 10 μg/ml) for scrape loading/dye transfer) should not be cytotoxic under the conditions used to measure its effect on gap junctional communication.

In order to ascertain whether FRAP analysis could detect gap junctional intercellular communication in primary rat glial cells, an experiment, as illustrated in Fig. 2, was performed. The results clearly demonstrate that 18 min after photobleaching of a single untreated cell, the fluorescence reappeared in coupled cells, but not in isolated cells. This is interpreted as indicating that the carboxyfluorescence dye was transferred, via gap junctions, to the photobleached cell. The lack of fluorescence in the isolated cell demonstrates that a new source of dye can be replaced only from gap junctionally coupled

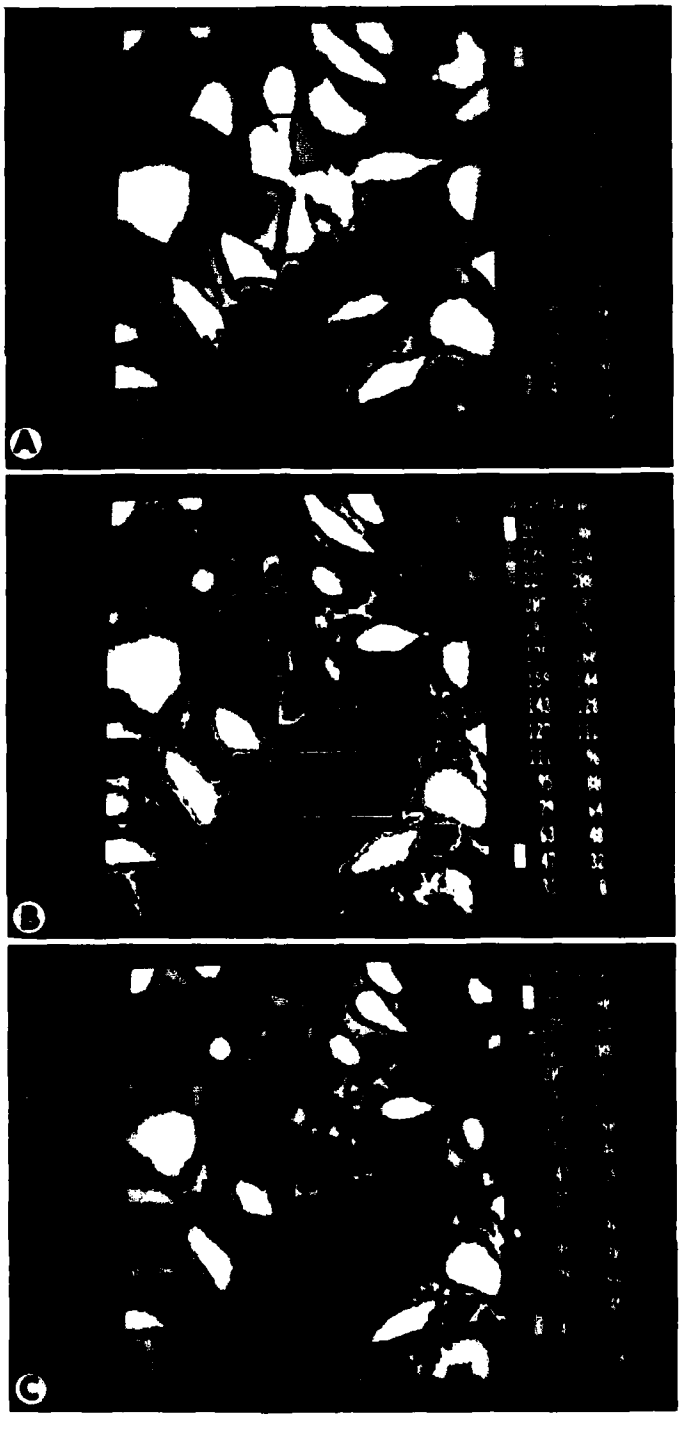

A
B
C

TABLE 1

RESULTS OF FRAP ANALYSIS ON DIELDRIN-TREATED RAT GLIAL CELLS

Treatment	Number of cells	Percentage of cells with fluorescence recovery
Control	25	100
24 hr pretreatment with dieldrin (7 μg/ml)	40	0.0
1 hr pretreatment with dieldrin (7 μg/ml)	6	0.0
10 min pretreatment with dieldrin (7 μg/ml)	43	0.0
1 hr pretreatment with dieldrin plus 4 hr post-treatment minus dieldrin	20	100
1 hr pretreatment with dieldrin plus 1 hr post-treatment minus dieldrin	6	83

unphotobleached cells. This figure is typical of 25 other samples in the same dish.

To test if dieldrin could inhibit gap junctional intercellular communication in rat glial cells, the experiment showed that 24 hr treatment with 7 μg/ml dieldrin prevented gap junction-mediated transfer of the fluorescent dye (Table 1).

Results in Table 1 also demonstrate that a 1-hr treatment of 7 μg/ml dieldrin was sufficient to inhibit gap junctional communication as measured by FRAP analysis. This result was typical of six randomly chosen coupled cells.

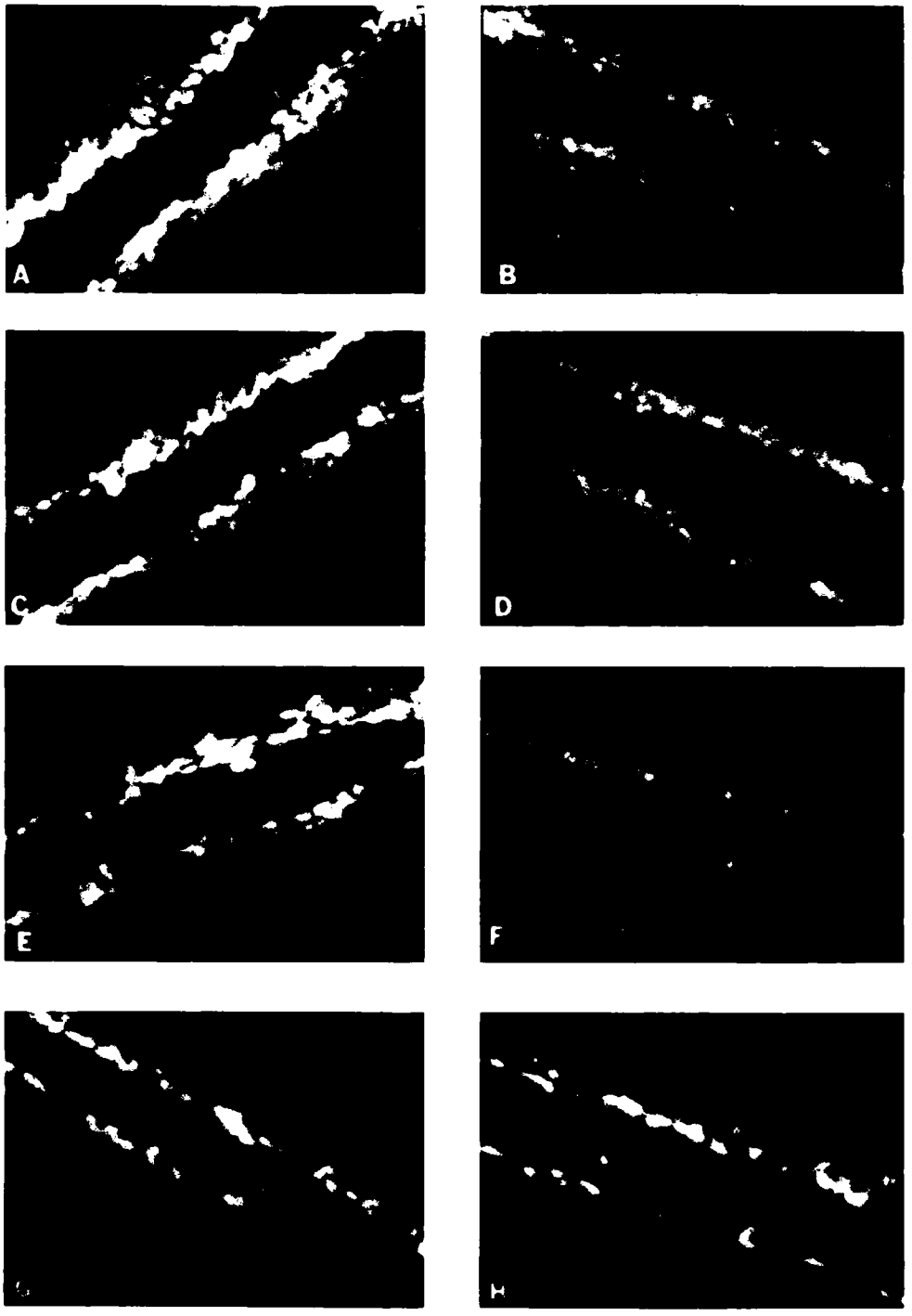
To determine if shorter treatment times could inhibit gap junctional communication a series of experiments were performed. The data in Table 1 illustrate that a 10-min exposure to 7 μg/ml dieldrin was sufficient to inhibit communication. Again, this result was repeated in 43 other randomly chosen coupled cells.

Since it is important to determine if the dieldrin inhibition of intercellular communication is either irreversible or reversible, cells were treated with dieldrin (7 μg/ml) for 1 hr and allowed to "recover" for 1 and 4 hr after the dieldrin was removed. Cells were washed twice and placed in fresh non-dieldrin containing medium. Data in Table 1, representative of *20* random samples, show that a 4-hr post-treatment time was sufficient for the reestablishment of gap junctional communication. In addition, results also indicate that 1 hr seems sufficient for these rat glial cells to reestablish gap junctional communication in six randomly chosen cells.

Scrape Loading Results

The results obtained from scrape loading/dye transfer assay are shown in Figs. 3 and 4. Quantitative analysis of the dose–response data, as described under Materials and Methods, indicates a direct correlation between the extent of blockage of gap junctional transfer of Lucifer yellow and the dieldrin concentration applied for a fixed period of 2 hr (Figs. 3 and 4A). When the cells were treated for variable exposure times with a fixed dose of dieldrin (7 μg/ml), the extent of junctional communication was inversly correlated with the duration of treatment (Fig. 4B; also data not shown). Complete inhibition of dye transfer was observed after approximately 50 min of initiating the treatment (Fig. 4B). The

FIG. 2. Restoration of fluorescence in photobleached, control primary rat glial cells. By comparing the images generated before photobleaching, when all cells were highly fluorescent as indicated by the false-color image in (A), with images produced 1 min (B) and 18 min (C) after bleaching, the recovery of fluorescence could be monitored. The image in (C) clearly shows the contacting, but not the isolated, cell regained its image after 18 min postbleaching. Image is ×300.


A
B
C
D
E
F
G
H

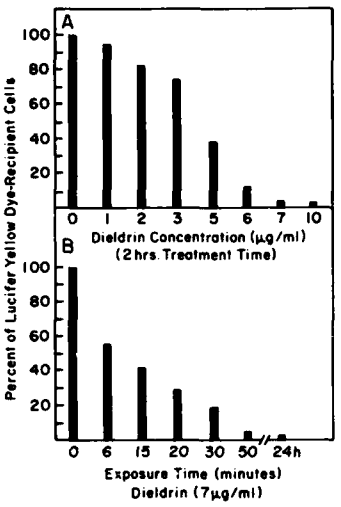


FIG. 4. (A) Inhibition of dye transfer in rat glial cells by dieldrin as detected by scrape loading/dye transfer assay. The method for quantitation is described under Materials and Methods. Dose–response effect of dieldrin on gap junction-mediated Lucifer yellow transfer. The cells were treated with variable concentrations of dieldrin for a fixed period of 2 hr. Panel (A) is a graphic representation of experiments shown in Fig. 3. (B) Time-course of the effect of dieldrin on dye transfer. A single treatment dose of dieldrin (7 μg/ml) was added to the cells for the indicated time periods followed by scrape loading of Lucifer yellow.

blockage of cell–cell communication by a single application of dieldrin was sustained for over 24 hr (Fig. 3). The inhibition of dye transfer was reversed when the cells were transiently exposed to dieldrin (7 μg/ml) for 1 hr then released and re-incubated in fresh medium. These cells resumed their control level of communication when examined 24 hr following the removal of dieldrin (data not shown).

DISCUSSION

There seem to be several conclusions resulting from the observations made during this study: (a) FRAP analysis and the scrape loading/dye transfer assay have verified earlier conclusions that gap junctional communication exists in rat glial cells (Orkand, 1977; Massa and Mugnaini, 1985); (b) dieldrin can inhibit gap junctional communication in rat glial cells, as measured by FRAP analysis, supporting previous observations that noncytotoxic levels of this toxic chemical inhibited gap junctional communication in Chinese hamster V79 and human teratocarcinoma cells as measured by metabolic cooperation and uridine transfer (Trosko *et al.*, 1987; Lin *et al.*, 1986); (c) FRAP analysis and scrape loading/dye transfer techniques, by corroborating the aforementioned studies, seem to be validated as a legitimate means to measure gap junctional intercellular communication; and (d) the effect of dieldrin inhibition of gap junctional communication is a reversible phenomenon.

Since gap junctional intercellular communication has been postulated to play a major role in the regulation of development, cell proliferation, regeneration, differentiation, homeostasis, and control of differentiated cell functions (Loewenstein, 1979; Pitts, 1980; Hertzberg *et al.*, 1981; Schultz, 1985; Larsen, 1983) in multicellular organisms, it seems logical to conclude that exogenous and endogenous chemical modulation of gap junction structure and/or function would have adaptive and nonadaptive consequences (Trosko and Chang, 1984). Many chemicals, which are known to be tumor promoters, have been demonstrated to be inhibitors of gap junctional communication (Jone *et al.*, 1985; Trosko *et al.*, 1982; Malcolm *et al.*, 1985).

One of those chemicals which is a tumor promoter of rat liver tumors and which inhibits gap junctional communication is dieldrin.

FIG. 3. Dose–response effect of dieldrin on junctional permeability in rat glial cells as measured by the scrape loading/dye transfer technique. The photomicrographs show Lucifer yellow transfer into contiguous cells pretreated with various concentrations of dieldrin. (A) Untreated control cells; (B–H) cells pretreated for 2 hr with 1, 2, 3, 5, 6, 7, and 10 μg dieldrin/ml, respectively.

What makes the dieldrin effect on the inhibition of gap junctional communication in rat glial cells relevant to these results is that dieldrin is also a known neurotoxin (Joy, 1982). In the former case, it has been postulated that when gap junctional communication is inhibited in tissues where a single carcinogen-initiated stem cell is repressed by surrounding normal cells, the initiated cell then clonally expands to form a tumor (Yotti *et al.*, 1979; Trosko *et al.*, 1983). In the latter case, although the role of gap junctional communication in neural cells has not been as well studied as the chemical neurotransmission form of intercellular communication, it is known to exist in brain tissue (Andrew *et al.*, 1981). In addition, two neurotransmitters, acetycholine and dopamine, have been shown to modulate gap junction function from several organisms (Iwatsuki and Petersen, 1978; Findlay and Petersen, 1982; Teranishi *et al.*, 1983; Piccolino *et al.*, 1984; Lasater and Dowling, 1985; Neyton and Trautmann, 1986). Since it would be hard to imagine, in evolutionary terms, that gap junctional communication plays no role in this highly specialized tissue, modulation of gap junction function in brain cells by dieldrin might be expected to play some role in its neurotoxicity.

Finally, as a note of speculation, one could imagine that, in the brain, chemical neurotransmission and gap junction transfer of ions and small molecular weight molecules comprise a highly coordinated and integrated intercellular communication network (Bennett *et al.*, 1985). Conceivably, gap junctional communication provides a means to regulate growth control and differentiation of premitotic cells, as well as a means to provide "nutrients" and regulatory signals to postmitotic neural cells. Endogenous and exogenous modulation of gap junctional communication in either pre- or postmitotic brain cells could have both adaptive, as well as toxic, consequences, depending on the nature of the inhibition.

REFERENCES

ANDREW, R. D., MACVICAR, B. A., DUDEK, F. E., AND HATTON, G. I. (1981). Dye transfer through gap junctions between neuroendocrine cells of rat hypothalamus. *Science* **211**, 1187–1189.

ASHWOOD-SMITH, M. J. (1981). The genetic toxicology of aldrin and dieldrin. *Mutat. Res.* **86**, 137–154.

ATKINSON, M. M., ANDERSON, S. K., AND SHERIDAN, J. D. (1986). Modification of gap junctions in cells transformed by a temperature-sensitive mutant of Rous sarcoma virus. *J. Membr. Biol.* **91**, 53–64.

ATKINSON, M., AND SHERIDAN, J. (1984). Decreased junctional permeability in cells transformed by three different viral oncogenes: A quantitative video analysis. *J. Cell Biol.* **99**, 401a.

AZARNIA, R., AND LOEWENSTEIN, W. R. (1984). Intercellular communication and the control of growth: X. Alterations of junctional permeability by the *src* gene. A study with temperature-sensitive mutant Rous sarcoma virus. *J. Membr. Biol.* **82**, 191–212.

AZARNIA, R., AND LOEWENSTEIN, W. R. (1987). Polyomavirus middle T antigen downregulates junctional cell to cell communication. *Mol. Cell. Biol.* **7**, 946–950.

BENNETT, M. V. L., AND GOODENOUGH, D. A. (1978). Gap junctions, electronic coupling and intercellular communication. *Neurosci. Res. Program. Bull.* **16**, 373–486.

BENNETT, M. V. L., SPRAY, D. C., AND HARRIS, A. L. (1981). Electrical coupling in development. *Amer. Zool.* **21**, 413–427.

BENNETT, M. V. L., ZIMERING, M. B., SPIRA, M. E., AND SPRAY, D. C. (1985). Interaction of electrical and chemical synapses. In *Gap Junctions* (M. V. L. Bennett and D. C. Spray, Eds.), pp. 355–366. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

CHANG, C. C., TROSKO, J. E., KUNG, H.-J., BOMICK, D., AND MATSUMURA, F. (1985). Potential role of the *src* gene product in the inhibition of gap junctional communication in NIH/3T3 cells. *Proc. Natl. Acad. Sci. USA* **82**, 5360–5364.

DAVIDSON, J. S., BAUMGARTEN, I. M., AND HARLEY, E. H. (1985). Effects of 12-*O*-tetradecanoylphorbol-13-acetate and retinoids on intercellular junctional communication measured with a citrulline incorporation assay. *Carcinogenesis* **6**, 645–650.

EL-FOULY, M. H., TROSKO, J. E., AND CHANG, C. C. (1987). Scrape-loading and dye transfer: A rapid and simple technique to study gap junctional intercellular communication. *Exp. Cell Res.* **168**, 422–430.

FINBOW, M. E., AND YANCEY, S. B. (1981). The roles of intercellular junctions. In *Biochemistry of Cellular Regulation* (M. J. Clemens, Ed.), Vol. 4, pp. 215–249. CRC Press, Boca Raton, FL.

FINDLAY, I., AND PETERSEN, O. H. (1982). Acetylcholine evoked uncoupling restricts the passage of lucifer

yellow between pancreatic acinar cells. *Cell Tissue Res.* **225,** 633–638.

FURSHPAN, E. J., AND POTTER, D. D. (1959). Transmission of the giant motor synapses of crayfish. *J. Physiol.* **145,** 289–325.

GUPTA, R. S., SINGH, B., AND STETSKO, D. K. (1985). Inhibition of metabolic cooperation by phorbol esters in a cell culture system based on adenosine kinase deficient mutants of V79 cells. *Carcinogenesis* **6,** 1359–1366.

HERTZBERG, E. L., LAWRENCE, T. S., AND GILULA, N. B. (1981). Gap junctional communication. *Annu. Rev. Physiol.* **43,** 479–491.

HOOPER, M. L. (1982). Metabolic cooperation between mammalian cells in culture. *Biochim. Biophys. Acta* **651,** 85–103.

ICPEMC Tast Group 5. (1984). Report of ICPEMC Task Group 5 on the differentiation between genotoxic and non-genotoxic carcinogens. *Mutat. Res.* **133,** 1–49.

ITO, N., TATEMATSU, M., NAKANISHI, K., HASEGAWA, R., TAKANO, T., IMAIDA, K., AND OGISO, T. (1980). The effects of various chemicals on the development of hyperplastic liver nodules in hepatectomized rats treated with *N*-nitrosodiethylamine or *N*-2-fluorenylacetamide. *Gann* **71,** 832–342.

IWATSUKI, N., AND PETERSON, O. H. (1978). Electrical coupling and uncoupling of exocrine acinar cells. *J. Cell Biol.* **79,** 533–545.

JONE, C., TROSKO, J. E., AYLSWORTH, C. F., PARKER, L., AND CHANG, C. C. (1985). Further characterization of the *in vitro* assay for inhibitors of metabolic cooperation in the Chinese hamster V79 cell line. *Carcinogenesis* **6,** 361–366.

JOY, R. M. (1982). Mode of action of lindane, dieldrin, and related insecticides in the central nervous system. *Neurobehav. Toxicol. Teratol.* **4,** 813–823.

KO, L., KOESTNER, A., AND WEICHSLER, W. (1980). Morphological characterization of nitrosource-induced glioma cell lines and clones. *Acta Neuropathol.* **51,** 23–31.

LARSEN, W. J. (1983). Biological implications of gap junction structure, distribution and composition. A review. *Tissue Cell* **15,** 645–671.

LARSEN, W. J., AND RISINGER, M. A. (1985). The dynamic life histories of intercellular membrane junctions. *Mod. Cell Biol.* **4,** 151–216.

LASATER, E. M., AND DOWLING, J. E. (1985). Dopamine decreases conductance of the electrical junctions between cultured retinal horizontal cells. *Proc. Natl. Acad. Sci. USA* **82,** 3025–3029.

LIN, Z.-X., KAVANAGH, T., TROSKO, J. E., AND CHANG, C. C. (1986). Inhibition of gap junctional intercellular communication in human teratocarcinoma cells by organochlorine pesticides. *Toxicol. Appl. Pharmacol.* **83,** 10–19.

LOEWENSTEIN, W. R. (1966). Permeability of membrane junctions. *Ann. N. Y. Acad. Sci.* **137,** 441–472.

LOEWENSTEIN, W. R. (1979). Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta* **560,** 1–65.

LOEWENSTEIN, W. R., AND KANNO, Y. (1966). Intercellular communication and the control of tissue growth. Lack of communication between cancer cells. *Nature (London)* **209,** 1248–1249.

MALCOLM, A. R., MILLS, L. J., AND MCKENNA, E. J. (1985). Effects of phorbol myristate acetate, phorbol dibutyrate, ethanol, dimethylsulfoxide, phenol and seven metabolites of phenol on metabolic cooperation between Chinese hamster V79 lung fibroblasts. *Cell Biol. Toxicol.* **1,** 265–283.

MASSA, P. T., AND MUGNAINI, E. (1985). Cell–cell junctional interactions and characteristic plasma membrane features of cultured rat glial cells. *Neuroscience* **14,** 695–709.

MCCANN, J., CHOI, E., YAMASAKI, E., AND AMES, B. N. (1975). Detection of carcinogen as mutagens in the salmonella/microsome test: Assay of 300 chemicals. *Proc. Natl. Acad. Sci. USA* **72,** 5135–5139.

MURRAY, A. W., AND FITZGERALD, D. J. (1979). Tumor promoters inhibit metabolic cooperation in cocultures of epidermal and 3T3 cells. *Biochem. Biophys. Res. Commun.* **91,** 395–401.

NEYTON, J., AND TRAUTMANN, A. (1986). Acetylcholine modulation of the conductance of intercellular junctions between rat lacrimal cells. *J. Physiol.* **377,** 283–295.

ORKAND, R. K. (1977). Glial cells. In *Handbook of Physiology*, Vol. 1, Section 1, *Cellular Biology of Neurons* (J. M. Brookhart, V. B. Mountcastle, E. R. Kandel, and S. R. Geiger, Eds.), pp. 855–875. Waverly Press, Baltimore.

PICCOLINO, M., NEYTON, J., AND GERSCHENFELD, H. M. (1984). Decrease of gap junctional permeability induced by dopamine and cyclic adenosine 3′-5′-monophosphate. *J. Neurosci.* **4,** 2477–2488.

PITTS, J. D. (1980). The role of junctional communication in animal tissues. *In Vitro* **16,** 1049–1056.

PROBST, G. S., MCMAHAN, R. E., HILL, L. E., THOMPSON, C. Z., EPP, J. K., AND NEAL, S. B. (1981). Chemically induced unscheduled DNA synthesis in primary rat hepatocytes: A comparison with bacterial mutagenicity using 218 compounds. *Environ. Mutat.* **3,** 11–32.

PURCHASE, I. F. H., LONGSTAFF, E., ASHBY, J., STYLES, J. A., ANDERSON, D., LEFEVREM, P. A., AND WESTWOOD, R. F. (1978). An evaluation of 6 short-term tests for detecting organic chemical carcinogens. *Brit. J. Cancer* **37,** 873–902.

SCHULTZ, R. M. (1985). Roles of cell to cell communication in development. *Biol. Reprod.* **32,** 27–42.

SUBAK-SHARPE, J. H., BURK, R. R., AND PITTS, J. D. (1969). Metabolic cooperation between biochemically marked mammalian cells in culture. *J. Cell. Sci.* **4,** 353–367.

TENNEKES, H. A., EDLER, L., AND KUNZ, H. W. (1982). Dose response analysis of the enhancement of liver tumor formation in CF-1 mice by dieldrin. *Carcinogenesis* **3,** 911–919.

TERANISHI, T., NEGISHI, K., AND KATO, S. (1983). Dopamine modulates *S*-potential amplitude and dye coupling between external horizontal cells in carp retina. *Nature (London)* **301,** 243–246.

TONG, C., FAZIO, M., AND WILLIAMS, G. M. (1981). Rat hepatocyte-mediated mutagenesis of human cells by carcinogenic polycyclic aromatic hydrocarbons but not organochlorine pesticides. *Proc. Soc. Exp. Biol. Med.* **167,** 572–575.

TROSKO, J. E., AND CHANG, C. C. (1984). Adaptive and nonadaptive consequences of chemical inhibition of intercellular communication. *Pharmacol. Rev.* **36,** 137s–144s.

TROSKO, J. E., CHANG, C. C., AND MEDCALF, A. (1983). Mechanisms of tumor promotion: Potential role of intercellular communication. *Cancer Invest.* **61,** 511–526.

TROSKO, J. E., JONE, C., AND CHANG, C. C. (1987). *In vitro* detection of chemicals which inhibit gap junctional-mediated intercellular communications: Aldrin, dieldrin and toxaphene as examples. *Biochem. Toxicol.* **1,** 83–93.

TROSKO, J. E., YOTTI, L. P., WARREN, S. T., TSUSHIMOTO, G., AND CHANG, C. C. (1982). Inhibition of cell–cell communication by tumor promoters. In *Carcinogenesis* (E. Hecker, N. E. Fusenig, W. Kunz, F. Marks, and H. W. Thielman, Eds.), Vol. 7, pp. 565–585. Raven Press, New York.

WADE, M., TROSKO, J. E., AND SCHINDLER, M. (1986). A fluorescence photobleaching assay of gap junction mediated cell–cell communication between human cells. *Science* **232,** 429–552.

YOTTI, L. P., CHANG, C. C., AND TROSKO, J. E. (1979). Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. *Science* **206,** 1089–1091.

Carcinogenesis vol.9 no.1 pp.135–139, 1988

Inhibition of gap junctional blockage by palmitoyl carnitine and TMB-8 in a rat liver epithelial cell line

Saw Yin Oh, Burra V.Madhukar and James E.Trosko

Department of Pediatrics and Human Development, College of Human Medicine, Michigan State University, East Lansing, MI 48824, USA

Exposure to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has been shown to inhibit gap junctional intercellular communication (GJIC) in many cell types *in vitro*. Using a scrape loading/dye transfer technique, TPA was shown to cause a dose-dependent and transient inhibition of GJIC in WB-F344, a normal rat liver epithelial cell line. Such a down-modulation of intercellular communication was found to be associated with an increase in protein kinase C (PKC) activity. Translocation of this activity to the particulate fraction occurred 10 min after exposure to 16 nM TPA and was consistent with the time course needed to inhibit GJIC. After 6 h exposure to TPA, essentially all the PKC activity was lost concurrent with the recovery of communication in these cells. During this time, the cells also became refractory to inhibition by further addition of TPA. Blockage of communication induced by TPA in WB cells was prevented by treating the cells with 23 μM palmitoyl carnitine for 1 h or 100 μM 8-*N,N*-(diethylamino)-octyl-3,4,5-trimethoxybenzoate for 30 min. The results indicate that TPA transiently modulates GJIC in WB cells and PKC activation is possibly involved in blockage of communication in these cells.

Introduction

Intercellular communication is considered an important cellular mechanism for regulating growth and differentiation (1–4). Thus blockage of the exchange of important 'signal' ions and molecules between normal communicating cells could lead to abnormal cell proliferation. Tumor promoting agents, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA*), have been shown to block gap junctional intercellular communication (GJIC) in various cell types (5–9). The precise biochemical mechanisms involved in the regulation of the gap junction, however, are not well understood. What is clear at present is the initial event of TPA action which involves binding to a specific, high affinity receptor (10–12), identified to be also the phospholipid-Ca^{2+}-dependent enzyme, protein kinase C (PKC) (13). This enzyme is now recognized to play a prominent role in signal transduction (14). Other studies have implicated activation of PKC with the TPA-inhibition of gap junctional communication (15–18). In this study, we investigated the effect of TPA on gap junctional communication in a normal adult rat liver epithelial cell line to assess the extent of PKC involvement in blockage of gap junctional communication in these cells.

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; GJIC, gap junctional intercellular communication; PKC, protein kinase C; PC, palmitoyl carnitine; TMB-8, 8-*N,N*-(diethylamino)octyl-3,4,5-trimethoxybenzoate; TCA, trichloroacetic acid; PDBu, phorbol dibutyrate.

Materials and methods

Cell culture

WB-F344 cells (obtained from Drs J.W.Grisham and M.S.Tsao of the University of North Carolina, Chapel Hill, NC), passages 10–25, were used. The cells were cultured in D medium, a modified Eagle's medium containing Earle's balanced salt solution with a 50% increase of vitamins and essential amino acids except glutamine, a 100% increase of non-essential amino acids (Gibco Laboratories, Grand Island, NY) and 1 mM sodium pyruvate, 5.5 mM glucose, 14.3 mM NaCl, 11.9 mM $NaHCO_3$, pH 7.3. The medium was supplemented with 5% fetal bovine serum and 50 μg/ml gentamicin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For all experiments, WB cells were seeded at 20% and grown to confluence. Treatments were made on cells 1–2 days post confluence. The culture medium was replaced with serum-free D medium prior to addition of test chemical(s) and incubated for the appropriate duration of the experiment.

Chemicals

TPA, palmitoyl carnitine (PC), 8-*N,N*-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) and histone type III-S were obtained from Sigma Chemicals, MO. TPA was dissolved in ethanol. PC and TMB-8 were dissolved in dimethylsulfoxide. Concentration of solvent in culture medium was 0.1%. Lucifer yellow and tetramethyl rhodamine dextran, mol. wt 10 000, were purchased from Molecular Probes Inc., Eugene, OR; [^{32}P]ATP (sp. act. 3000 Ci/mmol) was supplied by Amersham International, UK. All other biochemicals used in the investigation were of the highest purity available.

Measurement of GJIC

The method of scrape loading/dye transfer described by El-Fouly *et al.* (19) was used with a slight modification. Confluent cultures in 35 mm plates (1.2–1.6 × 10^6 cells) were rinsed several times with PBS and drained after treatment with the test chemicals. 2 ml of 0.05% Lucifer yellow in PBS was added to the plates and two or three scrape lines were made in the center of the monolayer with a surgical blade. After 3 min to allow dye uptake and transfer at room temperature, the cells were rinsed several times with PBS to remove excess dye and immediately examined under a Nikon epifluorescence phase microscope. Rhodamine dextran at 0.04% was occasionally added to the dye to verify that Lucifer yellow transfer was through the gap junctions.

Cells were fixed in 4% phosphate-buffered formalin and air-dried. The fixed cells were examined on the ACAS 470 fluorescence workstation (Meridian Instruments, Okemos, MI) with a laser beam (20). Using an appropriate computer program, a scan of the scrape-loaded cell image was generated and an integrated value of fluorescence intensity over a boxed area (78 mm × 100 mm) of the scrape line was obtained as a measure of the extent of dye transfer.

Preparation of partially purified cytosolic and membrane fractions

All operations were carried out at 4°C after incubation of the treated cells for the appropriate times. The cells were rinsed twice with PBS and twice with extraction buffer A containing 20 mM Tris–HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.33 M sucrose, 2 mM PMSF and 25 μg/ml leupeptin. Cells grown in two 150-cm^2 flasks were scraped into 4 ml of buffer A and disrupted in a glass–glass Dounce homogenizer (30 strokes). The homogenate was centrifuged at 100 000 *g* for 1 h and the supernatant was collected as the cytosolic fraction. The pellet was washed twice with buffer B (buffer A without sucrose) and resuspended in 4 ml of buffer B in a Dounce homogenizer (10 strokes). To solubilize PKC tightly associated with the membrane fraction, Nonidet P-40 was added to a final concentration of 1% and left for 40 min with gentle mixing. The solubilized suspension was centrifuged at 100 000 *g* for 1 h and the supernatant was used as the membrane (particulate) fraction. The cytosolic and membrane fractions were purified on a 1 ml packed bed volume of cellulose, DE-52 (Sigma) equilibrated with 20 mM Tris–HCl, pH 7.5. The column was washed with 15 ml of buffer B and after addition of sample, it was again washed with 6 ml of buffer B. PKC activity was eluted with 2 ml buffer B containing 0.1 M NaCl. Leupeptin at 25 μg/ml final concentration was added to the eluate.

PKC activity assay

Kinase activity was determined by the transfer of ^{32}P from [γ-^{32}P]ATP to histone based on the procedure described by Thomas *et al.* (21). The reaction was initiated

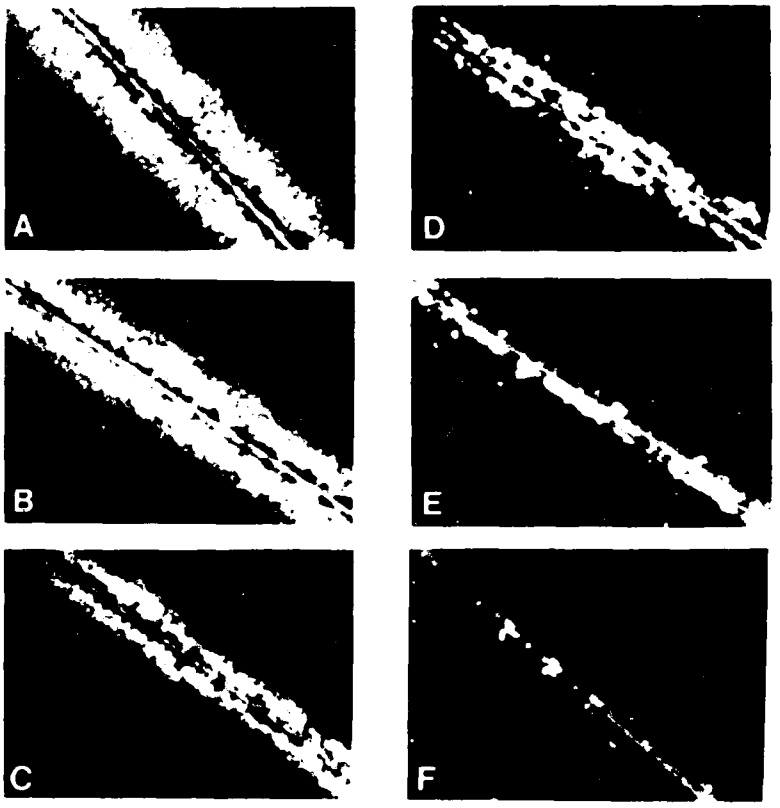


Fig. 1. Dye transfer in WB cells after 10 min incubation with different concentrations of TPA. Confluent cells in serum-free D medium were exposed to (**A**) 0.1% ethanol, (**B**) 1.6 nM TPA, (**C**) 3.2 nM TPA, (**D**) 8 nM TPA, (**E**) 16 nM TPA and (**F**) 80 nM TPA.

by the addition of 10 μg phosphatidylserine and 0.5 μg 1,2-diolein followed by 50 μl of enzyme preparation to reaction mixture containing 20 mM Tris–HCl, pH 7.5, 10 mM magnesium acetate, 0.4 mM $CaCl_2$, 100 μM [γ-^{32}P]ATP (~100 c.p.m./ pmol), 250 μg/ml histone III-S, 50 μg/ml leupeptin in a total volume of 200 μl. After incubation at 30°C for 3 min the reaction was terminated by adding 1 ml cold 25% trichloroacetic acid (TCA) and left overnight at 4°C. The precipitate was collected on a 0.45 μ Millipore filter. The filters were washed four times with 2 ml of 5% TCA, air-dried and the radioactivity quantitated in a liquid scintillation counter. Kinase activity was expressed as the difference between ^{32}P incorporation into histone in the absence of activators from that in the presence of activators. All assays were done in triplicate. Protein was determined by the method of Lowry *et al.* (22).

Results

Effect of TPA concentration on dye transfer

WB cells exposed to low concentrations of TPA (1.6 nM–160 nM) for 1 h blocked gap junctional communication. No, or slight, transfer of dye was observed in the TPA-treated cells while control cells exposed to 0.1% ethanol showed dye transfer in 8–12 rows of cells on either side of the scraped line. There was no difference between the ethanol control and untreated WB cells. Figure 1 shows the dose response of different concentrations of TPA on WB cells after 10 min exposure to the tumor promoter. A plot of the fluorescence intensity values against TPA concentrations confirmed the dose response (Figure 2). Very slight blockage was observed at a concentration of 1.6 nM TPA. There was no effect at 0.8 nM.

Time course of TPA effect on dye transfer

Blockage of intercellular communication by TPA in WB cells is reversible. Such a reversal of the inhibitory effect of TPA was time- and dose-dependent. Communication in WB cells treated with 160 nM TPA started to return as early as 3–4 h. By 6 h, the cells have re-established normal levels of communication in the continued presence of TPA in the medium. Similar experiments were done on WB cells using 16 nM TPA; at this concentration of TPA, reversal events occurred at a later time, about 6 h, and normal GJIC was restored by 12 h as shown in Table I.

The cells were exposed to a second treatment of 160 nM TPA for 10 min after a 12 h pre-treatment with the same dose of TPA. No inhibition of dye transfer was observed indicating that the pre-treated cells were refractory to the uncoupling effect of TPA. Another experiment using 16 nM TPA with a second treatment of 20 min after 18 h of pre-treatment confirmed the above observation.

Temporal relationship between PKC translocation and GJIC blockage

We determined the blockage of intercellular communication and PKC activation at different time intervals following treatment of WB cells with a single concentration of TPA (16 nM). Data presented in Table II clearly indicate that in untreated cells the majority of PKC activity was recovered from the cytosol. In TPA-treated cells, on the other hand, there was a translocation of PKC from cytosol to the membrane beginning 10 min post-treatment. PKC activity in the particulate fraction reached a maximum of about 5× that of control at 1 h, then fell below that of control level at 6 h. No activity was detected in the cytosolic fraction at this time point, indicating that almost all activity had been translocated. By 24 h virtually complete disappearance of the kinase was observed in WB cells under the conditions of the

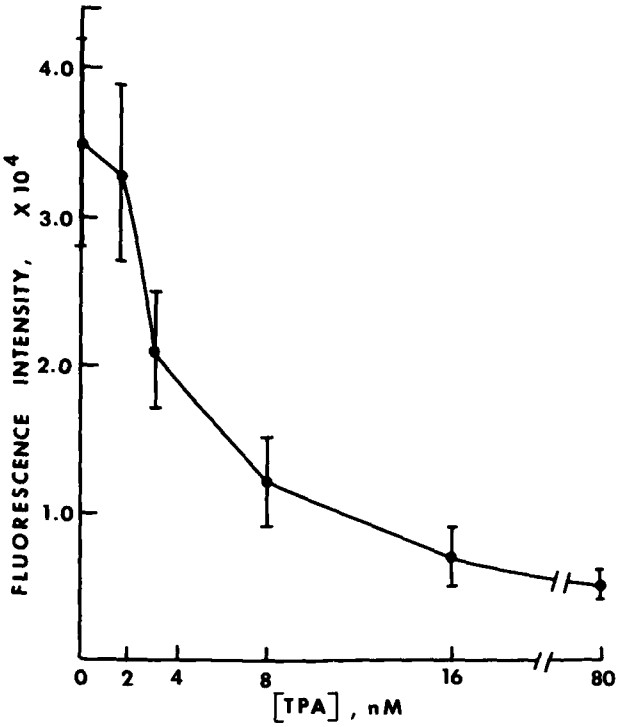


Fig. 2. Dose response relationship of TPA effect on GJIC in WB cells. The experiment was described in Figure 1. The fluorescence intensity of the dye as a measure of cell–cell communication was plotted against the concentration of TPA used. Each point represents the mean ± SE of at least 10 measurements.

Table I. Time course of TPA effect on dye transfer

Duration	Fluorescence intensity	% control
0	3.1 ± 0.9	100
10 min	0.8 ± 0.3	26
1 h	0.7 ± 0.2	22
6 h	1.6 ± 0.4	51
12 h	3.8 ± 0.7	96
18 h + TPA 20 min	2.9 ± 0.5	93

WB cells incubated in 16 nM TPA for different lengths of time. The value of the control at each time point was similar for the duration of the experiment. Each value is the mean ± SE of 10 scans.

Table II. Effect of TPA treatment on subcellular distribution of PKC in WB cells over a 24 h duration

TPA treatment duration	PKC activity (pmol/min/mg protein)		
	Particulate	Cytosol	Sum
Control	205	1360	1565
10 min	394	654	1048
30 min	637	320	957
1 h	1027	375	1402
6 h	90	0	90
12 h	100	0	100
24 h	22	0	22

Confluent flasks of WB cells were incubated in the presence of 16 nM TPA for different times. PKC activity was determined following DEAE–cellulose chromatography of cytosol and detergent-solubilized particulate fractions as described. Results are the average of two experiments at each time point. PKC assay was done in triplicate.

Table III. Effect of PC on TPA-induced blockage of dye transfer

Treatments	Fluorescence intensity × 10^4 ± SE	% control
Untreated	2.6 ± 1.0	
TPA	0.7 ± 0.4	27
PC, 2.3 μM	3.4 ± 0.9	
PC + TPA	1.5 ± 0.7	44
PC, 11.5 μM	2.5 ± 0.9	
PC + TPA	2.0 ± 1.0	80
PC, 23 μM	2.3 ± 0.6	
PC + TPA	2.4 ± 0.7	100

WB cells incubated with different concentrations of PC and 16 nM TPA for 1 h. PC was added followed by TPA. Each value is the mean ± SE of 10 scans.

Table IV. Effect of TMB-8 on TPA-induced blockage of dye transfer

Treatments	Fluorescence intensity × 10^4 ± SE	% control
Untreated	2.2 ± 0.7	
TPA	0.5 ± 0.3	22
TMB-8	1.8 ± 0.8	
TMB-8 + TPA	1.5 ± 0.5	83

WB cells incubated in 100 μM TMB-8 and 16 nM TPA for 30 min. TMB-8 was added followed by TPA. Each value is the mean ± SE of 10 scans.

experiment. A comparison with the data in Table I showed that translocation of PKC to the particulate fraction appears to correspond closely to blockage of GJIC in WB cells up to 1 h. By 6 h about 50% of the communication had returned, with normal communication level by 12 h corresponding to the loss of most of the detectable PKC activity in the cells. No detectable PKC activity was found in cells treated with TPA for 24 h.

PKC inhibitors, GJIC blockage and kinase translocation

To further verify that PKC may be involved in blockage by GJIC, two inhibitors of PKC, PC and TMB-8, were used. PC was reported to inhibit the activation of PKC in bovine heart (23,24) and in HL-60 cells (25,26). Table III shows the result of experiments done with three different concentrations of PC. PC was added, followed by TPA (16 nM) and left at 37°C for 1 h to determine if PC could inhibit the TPA-induced blockage of GJIC. Under the conditions of the experiment, PC did not block GJIC over the concentration range used. Some protection from TPA-induced blockage was observed with cells treated with 2.3 μM PC; when the concentration was raised to 11.5 μM almost complete protection was observed.

TMB-8, an intracellular calcium antagonist (27), has also been reported to inhibit PKC activation (28,29). Table IV shows the result of experiments using 100 μM TMB-8 in combination with 16 nM TPA incubated at 37°C for 30 min. TMB-8 alone did not appear to alter GJIC in these cells. When used with TPA, the cells were again almost completely protected by the TPA-induced blockage of GJIC.

We then compared the effect of the two inhibitors on PKC activation in WB cells under the conditions where the inhibitors protect the TPA-induced blockage of GJIC. Table V shows that PC, when co-administered with TPA, reduced translocation of PKC when compared with that translocated by TPA alone, indicating that PC could counteract the action of TPA mediated by PKC. No activity was detected in cells treated by PC alone,

Table V. Comparison of TPA, PC and TMB-8 effect on membrane-associated PKC (particulate fraction) in WB cells

Treatments	Particulate fraction PKC activity (pmol/min/mg protein)
Untreated	336
TPA 1 h	1479
PC 1 h	0
PC + TPA 1 h	559
Untreated	245
TPA 30 min	708
TMB-8 30 min	0
TMB-8 + TPA 30 min	0

Concentrations used were as follows: TPA, 160 nM; PC, 23 μM; TMB-8, 100 μM. Results are values obtained from two experiments using one 150-cm^2 flask of WB cells per treatment. Activity assay was done in triplicate.

suggesting a possible loss of the constitutive membrane-associated PKC in the treated cells. It is also possible that the effect of PC on the redistribution of PKC is not a simple unidirectional translocation of the enzyme from cytosol to membrane in these cells. In the TMB-8 experiment no activity was detected either in the control (TMB-8 alone) or in the treatment in combination with TPA.

Discussion

The results obtained in this study seem to implicate PKC activation in the TPA-induced GJIC blockage in WB cells. In addition, the ability of this liver cell culture to transfer Lucifer yellow rapidly makes this a useful *in vitro* system for the study of cell communication via gap junctions. nM quantities of TPA rapidly blocked GJIC in these cells with no apparent cytotoxicity and the degree of blockage varied with the dose and duration of TPA treatment. TPA-induced blockage of junctional communication was transient and corresponded well with the initial translocation of PKC from the cytosol to the membrane. This was followed by a decrease in membrane-associated PKC and a subsequent depletion of total PKC in 24 h. The process of desensitization of TPA has been well-noted in different cell types studied (30–32). Depletion of PKC activity by prolonged TPA treatment essentially corresponded to blockage of cell–cell communication in WB cells. This was further ascertained in cells pre-treated for 16 h where a second dose of TPA did not block GJIC. The recovery of TPA-induced blockage of cell communication in liver cells has also been reported by others (33,34).

Although it is possible that treatment with TPA has other effects on the cells, depletion of PKC seems the most likely reason given the above observations and the known involvement of PKC in phorbol ester responses. TPA has also been implicated in the induction of free radical generation (46,47) which could block junctional communication by an oxidative mechanism. CCl_4 which generates free radicals in hepatocyte culture causes uncoupling of the cells at 450 μM (48). H_2O_2 blocked junctional communication at μM concentrations in WB cells (J.Hewitt, personal communication). In monocytes, the amount of measurable superoxide induced by 10 nM TPA is in the order of 1 nmol/min/10^6 cells (44). The high amounts of free radicals required to block GJIC in the known cases makes it highly unlikely that nM concentrations of TPA used in this study would generate sufficient free radicals to block GJIC by an oxidative mechanism.

Thus the temporal relationship between the effect of TPA on GJIC and PKC suggests that PKC activation may be important in regulating gap junction function in WB cells. Furthermore, these results help to explain why TPA did not seem to inhibit metabolic co-operation in these WB cells (35). Since the metabolic co-operation assay, which is a gap-junction-dependent process (36), is carried out over a 3-day period before the 6-thioguanine-sensitive cells die from treatment, the reversal of inhibition of communication would restore metabolic co-operation, thereby leading to the death of 6-thioguanine-resistant cells.

PC and TMB-8, both known PKC inhibitors, counteracted TPA action on gap junctional blockage. PC was partially effective in preventing the blockage by TPA at 2.3 μM and almost completely abolished TPA effect at higher concentrations. These concentrations are still below those which have been reported to inhibit Na^+,K^+-ATPase (37). The activation of Na^+,K^+-ATPase by TPA is a relevant consideration as it could lead to accumulation of intracellular Na^+ which can increase mitochondrial release of Ca^{2+} (38). Since Na^+,K^+-ATPase stimulation requires μM concentration of TPA (39), it is unlikely that the enzyme was activated at the TPA concentrations used in this study. Thus the results with TMB-8 suggest that inhibition of TPA-induced blockage of GJIC involves mechanisms other than Ca^{2+} regulation. This compound, which was also reported to inhibit PKC (28) at concentrations used in this study (100 μM), could have blocked TPA action by inhibiting the activation of PKC in WB cells.

Some indirect evidence may be provided to answer whether PC or TMB-8 compete for binding to the phorbol ester receptor inhibiting TPA action on gap junctional blockage. PC at 30 μg/ml (69 nM) was shown to inhibit 50% of [^{3}H]phorbol dibutyrate (PDBu) binding in Friend leukemic cells and Chinese hamster V79 cells (40). This concentration was much higher than the dose effective for preventing TPA-induced gap junctional blockage in WB cells. Addition of either PC or TMB-8 30 min prior to addition of TPA did not prevent the blockage more effectively (data not shown). Moreover, TPA was 100-fold more active than PDBu for binding to cells. Hence its ability to displace PC or TMB-8 makes interaction at the receptor level relatively unlikely to explain the action of these compounds. PC being hydrophobic could readily insert into the cell membrane (41) and interact with enzyme(s) requiring lipid substrates or co-factors (23,42,43) or alter fluidity of the plasma membrane and hence enzyme activities.

PC partially inhibited the activation of PKC induced by TPA in intact WB cells. It is interesting to note that PC itself causes the disappearance of the constitutive pool of membrane-associated PKC. This loss could be due to the inactivation of the enzyme by PC or its translocation into the cytosol as has been observed with the effect of conA (44). Inactivation of the membrane-associated PKC activity by PC has been reported in intact pancreatic acini cells (45). It is conceivable that a distinct pool of membrane-associated PKC may be involved in the TPA-induced blockage of GJIC and PC or that similar compounds counteracted the action of the enzyme to prevent the blockage. TMB-8 completely depleted PKC in the particulate fraction, even in the presence of TPA, suggesting that translocation from the cytosol, if any, was effectively blocked. Further studies on the mechanism(s) of action of these compounds are required to understand more fully the TPA-induced blockage of cell–cell communication.

Acknowledgements

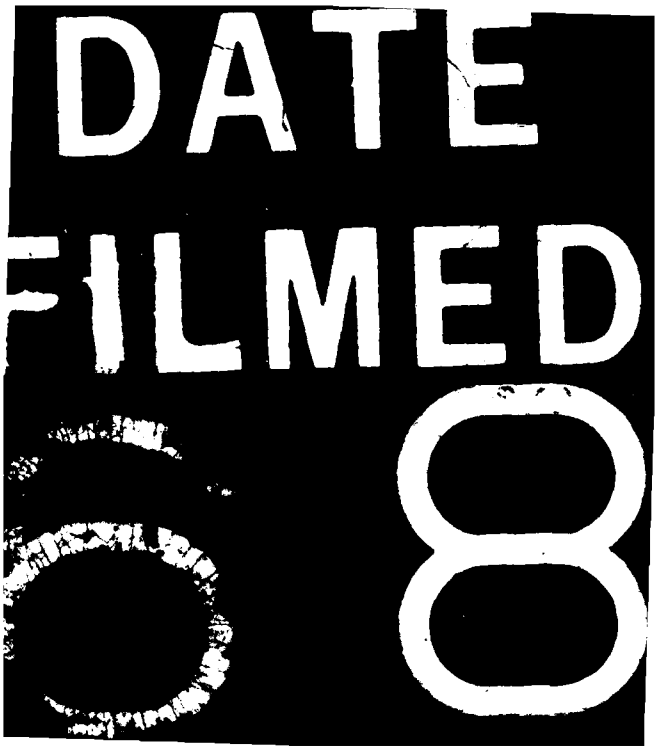
We thank Dr Alfred H.Merrill, Department of Biochemistry, Emory University School of Medicine, Atlanta, GA, for his suggestions and reviewing the manu-

script. The authors wish to acknowledge the technical assistance of Mrs Beth Lockwood and the word processing skills of Mrs Darla Conley. This work was supported by a United States Air Force Office of Scientific Research Grant No. AFOSR 86-0084.

References

1. Loewenstein,W.R. (1979) Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta*, **560**, 1–65.
2. Schultz,R.M. (1985) Roles of cell to cell communication in development. *Biol. Reprod.*, **32**, 27–42.
3. Lo,C.W. (1985) Communication compartmentation and pattern formation in development. In Bennett,M.V.L. and Spray,D.C. (eds), *Gap Junctions*. Cold Spring Harbor Laboratory Press, NY, pp. 251–263.
4. Pitts,J.D. and Finbow,M.E. (1986) The gap junction. *J. Cell Sci.*, **4**, 239–266.
5. Yotti,L.P., Chang,C.C. and Trosko,J.E. (1979) Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. *Science*, **208**, 1089–1091.
6. Murray,A.W. and Fitzgerald,D.J. (1979) Tumor promoters inhibit metabolic cooperation in co-cultures of epidermal and 3T3 cells. *Biochem. Biophys. Res. Commun.*, **91**, 395–401.
7. Enomoto,T., Sasaki,Y., Shiba,Y., Kanno,Y. and Yamasaki,H. (1981) Tumor promoters cause a rapid and reversible inhibition of the formation and maintenance of electrical cell coupling in culture. *Proc. Natl. Acad. Sci. USA*, **78**, 5628–5632.
8. Chang,C.C., Trosko,J.E., Kung,H.J., Bombick,D.W. and Matsumura,F. (1985) Potential role of the src gene product in the inhibition of gap-junctional communication in NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA*, **82**, 5360–5364.
9. Kavanagh,J.J., Chang,C.C. and Trosko,J.E. (1986) Characterization of a human teratocarcinoma cell assay for inhibitors of metabolic cooperation. *Cancer Res.*, **46**, 1359–1366.
10. Niedel,J.E., Kuhn,L.J. and Vandenbark,G.R. (1983) Phorbol diester receptor co purifies with protein kinase C. *Proc. Natl. Acad. Sci. USA*, **80**, 36–40.
11. Blumberg,P.M., Jaken,S., Konig,B., Sharkey,N.A., Leach,K.L., Jeng,A.Y. and Yeh,E. (1984) Mechanism of action of the phorbol ester tumor promoters: specific receptors for lipophilic ligands. *Biochem. Pharmacol.*, **33**, 933–940.
12. Ashendel,C.L. (1985) The phorbol ester receptor: a phospholipid-regulated protein kinase. *Biochim. Biophys. Acta*, **822**, 219–242.
13. Nishizuka,Y. (1986) Studies and perspectives of protein kinase C. *Science*, **233**, 305–312.
14. Nishizuka,Y. (1984) The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature*, **308**, 693–698.
15. Davidson,J.S., Baumgarten,I.M. and Harley,E.H. (1985) Studies on the *mechanism of phorbol ester-induced inhibition of intercellular junctional communication*. *Carcinogenesis*, **6**, 1353–1358.
16. Enomoto,T. and Yamasaki,H. (1985) Rapid inhibition of intercellular communication between BALB c 3T3 cells by diacylglycerol, a possible endogenous functional analogue of phorbol esters. *Cancer Res.*, **45**, 3706–3710.
17. Garner,H.St C. and Murray,A.W. (1985) Diacylglycerol inhibits gap junctional communication in cultured epidermal cells: evidence for a role of protein kinase C. *Biochem. Biophys. Res. Commun.*, **126**, 1109–1113.
18. Muir,J.G. and Murray,A.W. (1986) Mimicry of phorbol ester responses by diacylglycerols. Differential effects on phosphatidylcholine biosynthesis, cell–cell communication and epidermal growth factor binding. *Biochim. Biophys. Acta*, **885**, 176–184.
19. El-Fouly,M.H., Trosko,J.E. and Chang,C.C. (1987) Scrape-loading and dye *transfer*: a rapid and simple technique to study gap junctional intercellular communication. *Exp. Cell Res.*, **168**, 422–430.
20. Schindler,M., Trosko,J.E. and Wade,M.H. (1987) Fluorescence photobleaching assay of tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate inhibition of cell–cell communication. *Methods Enzymol.*, **141**, 439–447.
21. Thomas,T.P., Gopalakrishna,R. and Anderson,W.B. (1987) Hormone- and tumor promoter induced activation or membrane association of protein kinase C in intact cells. *Methods Enzymol.*, **141**, 399–411.
22. Lowry,O.H., Rosenbrough,N.J., Farr,A.L. and Randall,R.J. (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
23. Katoh,N., Wrenn,R.W., Wise,B.C., Shoji,M. and Kuo,J.F. (1981) Substrate proteins for calmodulin sensitive and phospholipid sensitive Ca^{2+} dependent protein kinases in heart, and inhibition of their phosphorylation by palmitoyl-carnitine. *Proc. Natl. Acad. Sci. USA*, **78**, 4813–4817.
24. Wise,B.C., Glass,D.B., Jen Chou,C.-H., Raynor,R.L., Katoh,N., Schatzman, R.C., Turner,R.S., Kebler,R.F. and Kuo,J.F. (1982) Phospholipid-sensitive Ca^{2+} dependent protein kinase from heart. II. Substrate specificity and inhibition by various agents. *J. Biol. Chem.*, **257**, 8489–8495.
25. Heilman,D.M., Barnes,K.C., Kinkade,J.M., Vogler,W.R., Shoji,M. and Kuo,J.F. (1983) Phospholipid sensitive Ca^{2+} dependent protein phosphorylation system in various types of leukemic cells from human patients and in human leukemic cell lines HL-60 and K562 and its inhibition by alkylphospholipid. *Cancer Res.*, **43**, 2955–2961.
26. Nakaki,T., Mita,S., Yamamoto,S., Nakadate,T. and Kato,R. (1984) Inhibition by palmitoyl carnitine of adhesion and morphological changes in HL-60 cells induced by 12-*O*-tetradecanoylphorbol-13-acetate. *Cancer Res.*, **44**, 1908–1912.
27. Mix,L.L., Dinerstein,R.J. and Villereal,M.L. (1984) Mitogens and melittin stimulate an increase in intercellular free calcium concentration in human fibroblasts. *Biochem. Biophys. Res. Commun.*, **119**, 69–75.
28. Kojima,I., Kojima,K. and Rasmussen,H. (1985) Mechanism of inhibitory action of TMB-8 [8-*N*,*N*-(diethylamino)octyl-3,4,5-trimethoxybenzoate] on aldosterone secretion in adrenal glomerulosa cells. *Biochem. J.*, **232**, 87–92.
29. Sawamura,M. (1985) Inhibition of protein kinase C activation by 8-*N*,*N*-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), an intracellular Ca^{2+} antagonist. *Kobe J. Med. Sci.*, **31**, 221–232.
30. Rodriguez-Pena,A. and Rozengurt,E. (1984) Disappearance of Ca^{2+}-sensitive, phosphoplipid-dependent protein kinase activity in phorbol ester-treated 3T3 cells. *Biochem. Biophys. Res. Commun.*, **120**, 1053–1059.
31. Ballester,R. and Rosen,O.M. (1985) Fate of immunoprecipitable protein kinase C in GH_3 cells treated with phorbol 12-myristate 13-acetate. *J. Biol. Chem.*, **260**, 15194–15199.
32. Hovis,J.G., Stumpo,D.J., Halsey,D.L. and Blackshear,P.J. (1986) Effects of mitogens on ornithine decarboxylase activity and messenger RNA levels in normal and protein kinase C-deficient NIH-3T3 fibroblasts. *J. Biol. Chem.*, **261**, 10380–10386.
33. Yada,T., Rose,B. and Loewenstein,W.R. (1985) Diacylglycerol downregulates junctional membrane permeability. TMB-8 blocks this effect. *J. Membrane Biol.*, **88**, 217–232.
34. Mesnil,M., Montesano,R. and Yamasaki,H. (1986) Intercellular communication of transformed and non-transformed rat liver epithelial cells. Modulation by TPA. *Exp. Cell Res.*, **165**, 391–402.
35. Jone,C., Trosko,J.E. and Chang,C.C. (1987) Characterization of a rat liver epithelial cell line to detect inhibitors of metabolic cooperation. *In Vitro Cell Dev. Biol.*, **23**, 214–220.
36. Hooper,M.L. (1982) Metabolic cooperation between mammalian cells in culture. *Biochim. Biophys. Acta*, **651**, 85–103.
37. Wood,J.M., Bush,B., Pitts,B.J.R. and Schwarz,A. (1977) Inhibition of bovine heart Na^+, K^+-ATPase by palmitoyl carnitine and palmitoyl-CoA. *Biochem. Biophys. Res. Commun.*, **74**, 677–684.
38. Carafoli,E. and Crompton,M. (1978) The regulation of intracellular calcium by mitochondria. *Ann. NY Acad. Sci.*, **307**, 269–284.
39. Blumberg,P.M. (1980) *In vitro* studies on the mode of action of the phorbol esters, potent tumor promoters: Part I. *CRC Crit. Rev. Toxicol.*, **8**, 153–197.
40. Tanaka,K., Ono,T. and Umeda,M. (1986) Inhibition of biological actions of 12-*O*-tetradecanoylphorbol-13-acetate by inhibitors of protein kinase C. *Gann*, **77**, 1107–1113.
41. Adams,R.J., Cohen,D.W., Gupte,S., Johnson,J.D., Wallick,E.T., Wang,T. and Schwartz,A. (1979) *In vitro* effects of palmitoyl carnitine on cardiac plasma membrane Na, K-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase and Ca^{2+} transport. *J. Biol. Chem.*, **254**, 12404–12410.
42. Bremer,J. and Norum,K.R. (1967) The effects of detergents on palmitoyl coenzyme A: carnitine palmitoyltransferase. *J. Biol. Chem.*, **242**, 1749–1755.
43. Gross,R.W. and Sobel,B.E. (1983) Rabbit myocardial cytosolic lysophospholipase: purification, characterization and competitive inhibition by L-palmitoyl carnitine. *J. Biol. Chem.*, **258**, 5221–5226.
44. Costa-Casnellie,M.R., Segel,G.B. and Lichtman,M.A. (1986) Signal transduction in human monocytes: relationship between superoxide production and the level of kinase C in the membrane. *J. Cell. Physiol.*, **129**, 336–342.
45. Brockenbrough,J.S. and Korc,M. (1987) Inhibition of epidermal growth factor binding in rat pancreatic acini by palmitoyl carnitine: evidence for Ca^{2+} and protein kinase C independent regulation. *Cancer Res.*, **47**, 1805–1810.
46. Goldstein,B.D., Witz,G., Amoruso,M., Stone,D.S. and Troll,W. (1981) Morphonuclear leukocyte superoxide anion radical production by tumor promoters. *Cancer Lett.*, **11**, 257–262.
47. Cerutti,P.A. (1985) Pro-oxidant states and tumor promotion. *Science*, **227**, 375–381.
48. Saez,J.C., Bennett,M.V.L. and Spray,D.C. (1987) Carbon tetrachloride at hepatotoxic levels blocks reversibly gap junctions between rat hepatocytes. *Science*, **236**, 967–969.

Received on August 4, 1987, revised on October 15, 1987, accepted on October 20, 1987


DATE
FILMED
8